# Abnormal Motor Function Persists Following Recovery from Perinatal Copper Deficiency in Rats[1,2]

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ABSTRACT What are the biochemical and behavioral consequences of perinatal copper deficiency? Pregnant Holtzman rats were fed a modified AIN-76A diet low in copper (0.34 mg Cu/kg and 42 mg Fe/kg) starting on gestation d 7. Seven rats received copper in their drinking water (20 mg Cu/L) (+Cu) and 7 drank deionized water (–Cu). Treatments did not affect litter size or pregnancy outcome. Compared with +Cu dams and a sample of +Cu male weanling [postnatal day (P)21] offspring, –Cu rats exhibited signs consistent with copper deficiency. P21 males were switched to a nonpurified copper-adequate diet and sampled biochemically after 3 mo and behaviorally after 3 and 6 mo of repletion (CuR). Compared with controls, CuR rats had lower brain copper and iron levels 3 and 6 mo after repletion; other biochemical differences were not detected. Behavioral assessments after 5 mo of repletion indicated a persistent impairment in motor function of CuR compared with control rats as evaluated by the accelerating rotorod procedure. These results suggest that permanent impairment to motor function can persist after long-term recovery from perinatal copper deficiency. J. Nutr. 134: 1984–1988, 2004.

KEY WORDS: • *copper deficiency* • *rats* • *motor function* • *behavior* • *copper repletion*

Development of full cognitive potential is a recognized goal of all human cultures. Although there are many external factors that can influence intellectual development, one factor, optimal neonatal nutrition, can be evaluated and implemented to achieve success. Wauben and Wainwright (1) reviewed the critical role nutrition plays in behavioral development. In particular, adequate protein, carbohydrate, essential fatty acids, zinc, iron, choline, vitamin C, vitamin B-12, and folic acid were documented. It is quite probable that there are other key nutrients that affect central nervous system (CNS)[4] development.

Accumulating evidence supports the importance of copper for brain development and function. Clinical awareness of copper limitation is recognized in the altered hematological profile. The connection between copper limitation and anemia dates to 1848 and the disease chlorosis (2). Recognition of a role for copper in brain development was first appreciated in 1937 in ataxic sheep born to copper-deficient ewes (3). Laboratory rodents born to copper-deficient dams display many gross alterations to the CNS such as missing cerebella, focal necrosis of the cerebral cortex, and lesions in the corpus striatum (4). Humans missing the P-type ATPase, ATP7A, cannot transport copper through cells properly and develop Menkes disease, a fatal neurological degenerative disorder (5).

The biochemical mechanisms responsible for the neuropathology of copper deficiency remain elusive. It is generally believed that changes in cuproenzymes such as superoxide dismutase (SOD), cytochrome *c* oxidase (CCO), dopamine-$\beta$-monooxygenase (DBM), and peptidylglycine-$\alpha$-amidating monooxygenase (PAM) could explain abnormal development and function. However, elucidation of critical enzymes and mechanisms remains a work in progress.

Two major factors that influence the development of neuropathology after copper deficiency are the degree of copper limitation and the timing of the onset of deficiency. The requirement for dietary copper to support gestation and lactation is much greater than that needed for adult homeostasis (6). In fact, it is difficult to assess copper status in adults because few, if any, established biochemical markers of copper status change when dietary copper is restricted to conventional foods low in copper (7). Consequently, some question exists whether copper deficiency is really an important public health issue. However, copper deficiency is clearly an important concern for neonates. As reviewed previously, copper restriction during lactation in rodents lowers brain copper concentrations by 80%, whereas postlactation copper restriction lowers brain copper by only 30% (8). The reductions in organ copper levels are proportional to dietary copper intakes.

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[4] Abbreviations used: CCO, cytochrome *c* oxidase; CNS, central nervous system; +Cu, copper-adequate dams/pups; −Cu, copper-deficient dams/pups; CuR, copper-replete offspring; DBM, dopamine-$\beta$-monooxygenase; E, embryonic day; GFHNRC, USDA ARS Grand Forks Human Nutrition Research Center; P, postnatal day; PAM, peptidylglycine-$\alpha$-amidating monooxygenase; SOD, superoxide dismutase; UMD, University of Minnesota Duluth.

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Thus, both dietary copper concentration and the timing of inadequate copper intakes are critical.

Another important issue is recovery from copper deficiency. Are there long-term consequences of perinatal copper deficiency? Offspring of Sprague-Dawley rats subjected to perinatal copper deficiency exhibited a blunted auditory startle response 5 mo after repletion with copper (8). Biochemically all differences between copper-adequate controls and formerly copper-deficient males and females disappeared after 5 mo of repletion except for reduced brain copper in repleted rats in several brain regions.

The current study sought to confirm and extend previous work using a different rat strain, Holtzman, and a wider battery of tests to evaluate neurobehavioral function, including motor and sensory function, activity, memory, and avoidance behavior.

## MATERIALS AND METHODS

***Animal care and diets.*** The design of the study is outlined in **Figure 1**. Sperm-positive female Holtzman rats ($n$ = 18) were purchased commercially (Harlan Sprague Dawley). Rats were fed 1 of 2 dietary treatments, copper-deficient (–Cu) or copper-adequate (+Cu), consisting of a copper-deficient purified diet (Teklad Laboratories) and either low-copper drinking water or copper-supplemented drinking water, respectively. The purified diet was similar to the AIN-76A diet (9,10). It contained the following major components (g/kg diet): sucrose, 500; casein, 200; cornstarch, 150; corn oil, 50; cellulose, 50; modified AIN-76 mineral mix, 35; AIN-76A vitamin mix, 10; DL-methionine, 3; choline bitartrate, 2; and ethoxyquin 0.01. Cupric carbonate was omitted from the AIN-76 mineral mix. The purified diet contained 0.34 mg Cu/kg and 42 mg Fe/kg by chemical analysis. Rats administered the –Cu treatment drank deionized water, whereas +Cu treatment groups drank water that contained 20 mg Cu/L through the addition of $CuSO_4$. Before treatment and during repletion, rats consumed nonpurifed diet, Purina Laboratory Rodent Chow 5001 (Ralston Purina) and drank tap water. This diet contained 13.2 mg Cu/kg and 108 mg Fe/kg by analysis. Rats had free access to diet and drinking water. All rats were maintained at 24°C with 55% relative humidity with a 12-h light:dark cycle (0700–1900 h light). Dietary protocols were approved by the University of Minnesota (UMD) Animal Care Committee. Protocols for behavioral assessments and necropsy performed at the USDA ARS Grand Forks Human Nutrition Research Center (GFHNRC) were approved by its Animal Care and Use Committee.

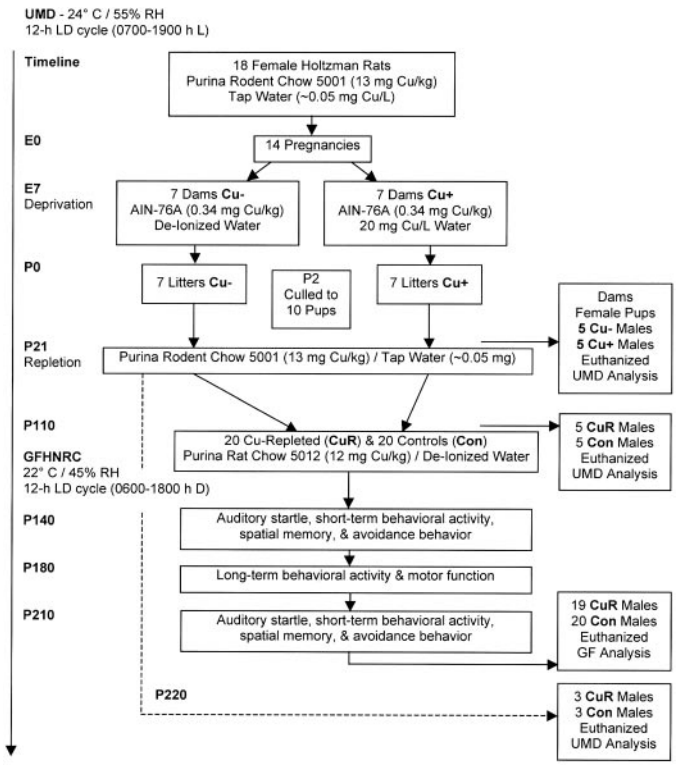


**FIGURE 1** Schematic representation of experimental design.

After 3 mo of repletion a cohort of 10 male rats, 5 controls (formerly +Cu) and 5 Cu-repleted (CuR) (formerly –Cu), were killed to evaluate copper status. The remaining males (representing all 14 litters) $n$ = 40, 20 controls and 20 CuR, were shipped to the GFHNRC in Grand Forks, ND for behavioral assessments and necropsy. Rats were coded by ear punch; their dietary history was not known to the investigators until assessments and necropsy were completed. At the GFHNRC, rats were housed in a climate-controlled room (temperature 22 ± 2°C; relative humidity 45 ± 10%) with a 12-h dark-light cycle (0600–1800 h dark), and fed a diet of Purina Laboratory Rodent Chow 5012 (Ralston Purina) and demineralized water for the remainder of the study.

Rats were housed at the GFHNRC for >30 d before behavioral assessments to permit adaptation to their new surroundings and diet, and handled individually for at least 5 min/wk to increase comfort with human contact and reduce anxiety during behavioral assessments.

For UMD data, rats were anesthetized with diethyl ether and decapitated. A sample of blood was collected to measure ceruloplasmin activity and hemoglobin. Livers and brains were removed and processed for biochemical analyses. For GFHNRC data, 6-mo-old rats were anesthetized with a ketamine hydrochloride and remifentanil hydrochloride solution, and blood was drawn via heart puncture. Rats were then decapitated and whole brain, liver, and right femur were removed and processed for biochemical analyses of copper and iron.

***Biochemical analyses.*** At UMD, plasma from hematocrit tubes was used to measure ceruloplasmin activity by following the oxidation of *o*-dianisidine (11). Portions of liver and diet (~1 g), and the entire cerebral cortex were weighed to the nearest 0.1 mg and wet-digested with 4 mL of concentrated $HNO_3$ (Trace Metal Grade, Fisher Scientific); the residue was brought to 4.0 mL with 0.1 mol/L $HNO_3$. Samples were then analyzed for total copper and iron by flame atomic absorption spectroscopy (Model 2380, Perkin-Elmer). The method was verified using a certified standard, U.S. National Bureau of Standards 1577 bovine liver. At GFHNRC, hematocrit and hemoglobin were determined by automated cell counter (Cell-Dyn model 3500CS, Abbott). Femurs were cleaned with cheesecloth (Labcraft, CMS) to the periosteal surface and, along with other tissues, freeze-dried in a triphilizer (FTS System) at 25°C for 6–7 d. Subsequently, all samples were digested (160°C) with 16 mol/L ultrapure nitric acid (VWR Scientific) in Teflon tubes (Nalge), and then wet-ashed with a 1:3 solution of 16 mol/L $HNO_3$:30% $H_2O_2$ (hydrogen peroxide, superoxol stabilized, VWR Scientific). All samples were diluted 1:10 with 1 mol/L HCL (Vycor, double-distilled, GFS Chemicals). Samples were then analyzed for copper and iron using an inductively coupled argon plasma atomic emission spectrometer (ICAP model Optima 330DV, Perkin-Elmer). The method was checked with certified standards, U.S. National Bureau of Standards 1577B bovine liver and 1567A wheat flour.

***Behavioral assessments.***[5] Auditory startle, short-term behavioral activity, spatial memory, and avoidance behavior were each

[5] Detailed descriptions of equipment and procedures used for behavioral assessments, and tables showing results of all behavioral assessments are available as supplemental data with the online posting of this paper at www.nutrition.org.

evaluated twice, at ~P140 and P210, and long-term behavioral activity and motor function were each evaluated once, at ~P160 and P180, respectively. All behavioral assessments were done under red-light conditions between 0800 and 1600 h, during the dark phase of the light-dark cycle and at least 2 h after the onset of dark.

*Auditory startle* was evaluated with a Coulbourn Instruments Acoustic Startle System. After a 5-min habituation period, a 110-dB scale A white noise burst (50 ms duration, 2 ms rise/fall time) was presented during 25 trials (30-s mean intertrial interval) and peak and mean response amplitudes (g), latency to peak response amplitude (s), and mean duration of response (s) were recorded.

*Short-term spontaneous behavioral activity* was evaluated for 30 min with a Coulbourn Instruments Tru Scan Photobeam Activity System. Analyzed data included total number, distance (cm) and time (s) of all movements, number and time (s) of stereotypic (repeated) and circular movements, number of vertical movements (rearing) and nose pokes, and number of entries and time (s) spent in different areas of the arena.

*Spatial memory* was evaluated with a Coulbourn Instruments 8-arm radial maze. Arms/feeders were baited with precision 20 mg Noyes Dustfree Sucrose Reward Tablets (PJFSC-0020, Research Diets). Memory assessment generally followed the traditional procedure described by Olton and Samuelson (12). After 5 d of training with restricted access to previously visited arms, performance was evaluated during 3 consecutive 5-min trials. Analyzed data included number of entries, reentries, time (s) spent in each arm, and food consumed.

*Avoidance behavior* was evaluated with the elevated plus-maze procedure described by Pellow et al. (13). Number of entries and time (s) spent in the open and closed arms of the maze were determined during 2 consecutive 5-min trials. For half of the rats, the first trial began when the rat was placed in the center of the maze facing an open arm and the second trial began when the rat was placed in the center of the maze facing a closed arm; starting orientation was reversed for the other half of the rats.

*Long-term spontaneous behavioral activity* in the rat's home cage was evaluated for 44 h (2 consecutive 22-h periods) with a Coulbourn Instruments Infrared Home Cage Activity Monitoring System. Number and time (s) spent involved in large (sustained) and small (brief) body movements in 3 dimensions were determined.

*Motor function* was evaluated with the accelerating rotorod procedure described by Tilson (14). A test trial began when the rat was placed on the rod facing the back of the chamber and ended when it fell to the floor, with time on the rod determined by a technician using a stopwatch. After training on a stationary rod and then on a rod rotating at the constant rate of 1 rpm, time (s) on rod (latency to fall, 210 s maximum) and speed attained in rpm were recorded as rod rotation increased from 1 to 20 rpm (1 rpm increase every 10 s); 3 trials were run each day for 5 consecutive days.

***Statistics.*** At UMD, dietary treatment effects were evaluated by Student's *t* test after variance equality was tested, $\alpha = 0.05$, or by factorial ANOVA (Statview 4.5, Abacus Concepts). At GFHNRC, biochemical data were analyzed for treatment effects with Student's *t* test, $\alpha = 0.05$. Behavioral data meeting parametric distribution assumptions were analyzed with Student's *t* test (one-session) or mixed-model ANOVA (multiple sessions), $\alpha = 0.05$; the Tukey-Kramer test was used for subsequent contrasts. Nonparametric behavioral data (Kolmogorov-Smirnov, $P < 0.01$) were analyzed as ranked data with Wilcoxon 2-Sample or *F*-approximation to Friedman's test for 1 and multiple sessions, respectively (SAS 8.02, SAS Institute).

## RESULTS

All 14 pregnant rats delivered healthy pups ($n = 190$) with an average litter size of 13.6. After culling litter size to 10 pups, there were 4 deaths, all from –Cu dams, before sampling and the start of repletion at P21.

***Biochemical and clinical variables.*** Compared with +Cu dams, –Cu dams had near total loss of ceruloplasmin diamine oxidase activity, a 75% lower liver copper concentration, and a robust increase in liver iron concentration after the 5-wk treatment period (2 wk of gestation and 3 wk of lactation) (**Table 1**). Body weight and hemoglobin concentration were not affected by diet history of the dams. Male pups from –Cu dams exhibited signs consistent with copper deficiency compared with +Cu offspring at P21 (Table 1). These features included lower ($P < 0.05$) body weight, hemoglobin, ceruloplasmin, and liver copper concentration. Liver iron content was not altered. Brain copper concentration was 87% lower in cortex samples from –Cu pups compared with +Cu pups. Interestingly, we also detected a significantly lower iron concentration in the cortex of –Cu rats. The absolute difference is likely less than measured because these organs were not perfused, and the iron contamination from blood would be greater in the +Cu rats because the hemoglobin concentration was higher (Table 1).

After 3 mo of copper repletion, most copper status indicators in the former –Cu offspring did not differ from control values (**Table 2**). Specifically, copper-repleted (CuR) rats retained at UMD were not longer lighter; they were not anemic and did not have reductions in liver copper or ceruloplasmin activity. However, even after 3 mo of copper repletion, there was a persistent and significant reduction in cortex copper and iron concentrations (Table 2). In fact, brain copper was 38% lower in CuR rats compared with controls. Another sample of male rats ($n = 3$) retained at UMD were killed after 6 mo of repletion. Compared with control values of brain copper, 2.59 ± 0.05 μg/g (40.7 nmol/g), CuR rats had a 32% lower values, 2.01 ± 0.09 μg/g (31.6 nmol/g), $P < 0.01$. We did not detect any other biochemical differences between these 2 treatment groups.

**TABLE 1**

*Characteristics of rat dams and 21-d-old male pups after perinatal copper deficiency*[1]

| | Dams | | Pups | |
|---|---|---|---|---|
| Measure | Copper-Adequate | Copper-Deficient | Copper-Adequate | Copper-Deficient |
| Body weight, *g* | 363 ± 6.4 | 355 ± 21.9 | 65.4 ± 3.0 | 50.7 ± 4.9* |
| Hemoglobin, *g/L* | 174 ± 6.8 | 163 ± 4.4 | 68.6 ± 4.8 | 49.5 ± 6.3* |
| Ceruloplasmin, *units/L* | 350 ± 24.4 | 3.06 ± 1.48* | 108 ± 6.48 | 0.01 ± 0.17* |
| Liver copper,[2] *μg/g* | 3.13 ± 0.21 | 0.78 ± 0.11* | 20.8 ± 5.13 | 0.39 ± 0.06* |
| Liver iron,[2] *μg/g* | 68.8 ± 2.5 | 109 ± 12.9* | 17.7 ± 2.09 | 22.8 ± 1.77 |
| Brain copper,[2] *μg/g* | ND | ND | 2.09 ± 0.34 | 0.28 ± 0.02* |
| Brain iron,[2] *μg/g* | ND | ND | 9.04 ± 0.93 | 6.57 ± 0.18* |

[1] Values are means ± SEM ($n = 7$ for dams and $n = 5$ for pups). * Different from +Cu, $P < 0.05$, by Student's *t* test. ND, not determined.
[2] One μg of copper = 15.7 nmol and 1 μg iron = 17.9 nmol.

**TABLE 2**

*Characteristics of male rats after 3 and 6 mo of copper repletion*[1,2]

| Measure | 3 mo | | 6 mo | |
|---|---|---|---|---|
| | Control | Copper-Repleted | Control | Copper-Repleted |
| Body weight, *g* | 634 ± 9.7 | 622 ± 21.6 | 743 ± 12.7 | 769 ± 16.5 |
| Hemoglobin, *g/L* | 158 ± 5.0 | 157 ± 2.0 | 155 ± 1.91 | 161 ± 1.21 |
| Ceruloplasmin, *units/L* | 218 ± 17.3 | 232 ± 23.7 | ND | ND |
| Liver copper,[3] *μg/g* | 3.92 ± 0.13 | 3.96 ± 0.04 | 16.1 ± 0.20 | 15.8 ± 0.23 |
| Liver iron,[3] *μg/g* | 91.6 ± 6.6 | 93.4 ± 6.3 | 588 ± 15.8 | 551 ± 16.7 |
| Brain copper,[3] *μg/g* | 2.54 ± 0.09 | 1.57 ± 0.04** | 12.9 ± 0.34 | 10.4 ± 0.27** |
| Brain iron,[3] *μg/g* | 14.4 ± 0.37 | 12.6 ± 0.21** | 101 ± 2.01 | 93.5 ± 1.45** |

[1] Values are means ± SEM (3 mo: $n = 5$; 6 mo: $n = 20$ for controls and $n = 19$ for copper-repleted rats). ** Different from control, $P < 0.01$, by Student's *t* test. ND, not determined.
[2] Rats were fed a nonpurified copper-adequate diet between P21 and P113 (3 mo) or P210 (6 mo).
[3] One μg of copper = 15.7 nmol and 1 μg iron = 17.9 nmol. Mineral data at 6 mo are expressed per dry weight.

Consistent with the above, analyses of whole brain conducted on a larger sample of 39 rats (19 CuR and 20 controls) at GFHNRC also found that CuR rats had lower copper and iron concentrations than control rats after 6 mo of repletion (Table 2). There were no other significant biochemical differences between the 2 treatment groups, nor were there any differences in body or tissue weights or body-tissue weight ratios (data not shown).

***Behavior.*** After 6 mo of repletion, the performance of CuR rats was inferior to that of controls on measures of motor function. On the rotorod task, both mean speed (**Fig. 2**, *panel A*) and mean time running on the rod (Fig. 2, *panel B*) were lower in CuR rats than in controls. Remarkably, other measures of neurobehavioral function, including auditory startle, short- and long-term behavioral activity, spatial memory, and avoidance behavior, did not indicate consistent differences between the 2 treatment groups.[5]

## DISCUSSION

Results of this study indicate very clearly that copper restriction during the last two thirds of gestation and throughout lactation results in rat offspring that are severely deficient in copper. However, when these very deficient rats are subsequently repleted through diet with a generous supply of copper, in excess of 200 μg/d, most characteristics of the deficient rats are rapidly reversed such that, by 3 mo of age, determining the most perceptible difference requires postmortem biochemical analysis of brain tissue. Results of the current study, therefore, are similar to those of studies we completed previously (8,15). In confirmation of earlier studies, brain copper was not restored to control levels even after 6 mo of dietary repletion (8).

In the current studies, one interesting new finding was that brain iron levels were lower after copper deficiency even after 3 mo of repletion and without evidence of anemia, thus ruling out blood contamination as an issue with nervous tissue. The physiologic importance of this small change in brain iron is not known. Frank iron deficiency does alter dopamine metabolism and related behaviors, including motor behavior, in developing animals (16). This difference in brain iron persisted even in the autopsy data of the rats after more than 6 mo.

It could be argued that the nutritional deprivation of copper in these studies was very severe and unlike what might occur in human populations. However, it should be noted that earlier studies by others found maturation defects in the brains of male rats when the dietary level of copper was 1.8 mg/kg (17). Therefore, a rather modest reduction in dietary copper can have a significant effect during the developmental period. This issue of the requirement for copper during early development compared with adulthood was recently underscored in a different rodent model (6). Another seminal study indicated that feeding a diet containing 2.6 mg/kg copper during repro-

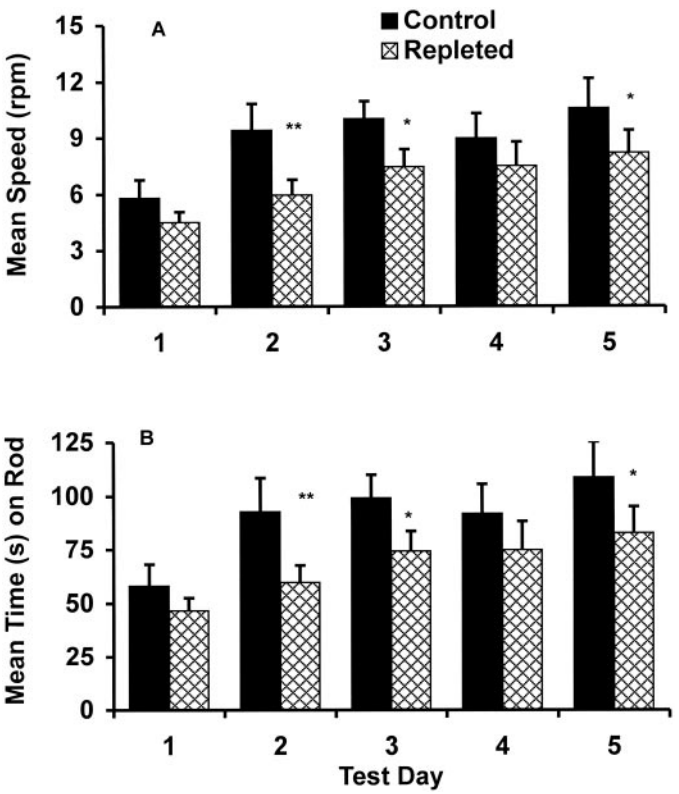


**FIGURE 2** Rotorod performance of male rats after 6 mo of copper repletion. *Panel A* shows the speed of the rotating rod before rats fell, in revolutions per minute (rpm); *panel B* shows the time on the rotating rod before they fell, in seconds (s). Values are means across trials ± SEM, $n = 20$ for controls and $n = 19$ for copper-repleted (CuR) rats. Asterisks indicate a difference from the control by Tukey-Kramer test: $^*P < 0.05$, $^{**}P < 0.005$.

duction and lactation compared with a diet with 6.7 mg/kg had an effect on brain copper in 6-mo-old offspring, whereas copper concentrations in other tissues were not affected (18). Brain copper concentrations were lower in both males and females in that experiment. Therefore, a diet considered adequate in copper, resulting in no biochemical manifestations in peripheral organs, was insufficient to saturate brain copper pools if fed during a critical period of development. Certain populations may be at greater risk for copper deficiency. For example, babies born to teenage mothers, whose copper intake is below the RDA and still require copper for their own growth, or premature infants, because of inappropriate transfer of copper from mother to baby, may require extra copper supplementation during the developmental period.

The cerebellum, a region in which cells develop postnatally in rats, would certainly be a region subject to nutritional insult. Thus, the abnormal motor behavior observed in the current experiment and in a previous study demonstrating abnormal foot splay are both consistent with changes in cerebellar function after copper deficiency (8). It was somewhat surprising that we found no blunted auditory startle response in the CuR rats given that there was a robust persistent alteration in startle in our previous study (8). In fact, a smaller cohort of CuR rats evaluated at GFHNRC previously did confirm altered startle (unpublished data). However, both that study and the previously published study used Sprague-Dawley rats. The current experiments used Holtzman rats. Although highly related genetically, perhaps differences in strain may explain the different outcomes in auditory startle. It is also possible that some other subtle differences in procedures may be responsible. It is worth noting that in the current study, 4 of 19 CuR rats, contrasted with only 1 of 20 controls, were excluded from statistical analysis due to the absence of a measurable startle response on a sufficient number of trials ($\geq$25%) to yield reliable data. Therefore, 21% of CuR rats had essentially no auditory startle response at all, a finding that is consistent with previous studies.

It is always difficult to pinpoint potential mechanisms that could be responsible for persistent abnormal behavior in otherwise healthy renourished animals. The rats in this experiment are no different. Because copper is required for ~10 different cuproenzymes, it is possible that limiting activity of any one of these might explain the altered persistent behavioral outcomes in these studies. One candidate that is being pursued is the cuproenzyme dopamine-$\beta$-monooxygenase. This protein is required for development of mammals as indicated in the embryonic lethality of null mice lacking DBM (19). A deficiency of norepinephrine during critical brain development might decrease synaptic connections and maturation of neurons, thus affecting behavioral, and in particular, motor responses. Some of these potential neurochemical defects may be irreversible. For example, there is a parallel situation that occurs after neonatal iron deficiency in which irreversible changes in dopaminergic function were demonstrated (20). It is also quite possible that the changes in other key enzymes such as SOD, PAM, or CCO may also play a role in the abnormal behavior observed in the repleted rats in this study. Further research is required to determine with greater precision the perinatal period most vulnerable to inadequate copper nutrition and the mechanistic basis of the persistent neurobehavioral abnormalities.

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